ACCEPTED MANUSCRIPT

SCREENING OF THE WHOLE EGG WHITE PROTEINS IN VARIABLE TYPES OF BIRDS

Al-Shuhaib MBS, Hashim HO, Ewadh MJ

DOI: 10.11598/btb.2019.26.2.812

To appear in : BIOTROPIA Vol. 26 No. 2 August 2019 Issue

Received date : 18 February 2017
Accepted date : 10 December 2018

SCREENING OF THE WHOLE EGG WHITE PROTEINS IN VARIABLE TYPES OF BIRDS

Mohammed Baqur S. Al-Shuhaib[1*], Hayder O. Hashim[2] and Mufeed J. Ewadh[3]

[1]Department of Animal Production, College of Agriculture, Al-Qasim Green University, Babil 51001, Iraq

[2]Department of Clinical Laboratory Sciences, College of Pharmacology, University of Babylon, Babil 51001, Iraq

[3]Branch of Clinical Biochemistry, College of Medicine, University of Babylon, Babil 51001, Iraq

[*]Corresponding author, e-mail: mohammed79@agre.uoqasim.edu.iq

Running title: SDS-PAGE of egg-white proteins

ABSTRACT

The comparative heterogeneity of the detailed, in-parallel protein composition data analysis for wide varieties of birds' egg white samples has not yet been fully defined. The main object of this research is to evaluate the extent of variability among more than 40 types of birds' egg white. To improve the perception of these biological fluids, the main phenotypes variations of egg white were evaluated using the discontinuous denaturing polyacrylamide gel electrophoresis (SDS-PAGE), Gradient SDS-PAGE, Native-PAGE, cellulose acetate electrophoresis, and reverse phase high-performance liquid chromatography (RP-HPLC). Though the latest techniques didn't show significant variability in terms of hydrophobicity, several electrophoretic differences of egg-white proteins were observed. As well, several unknown proteins in many egg white samples of different bird species were identified through electrophoretic experiments. So, it might be possible, as it shown in many cases of egg white samples, to provide a characterized assessment among birds only by using the available gel electrophoresis techniques. Also, this study provides a rapid snapshot for the initial identification of several unknown egg white protein components. According to our knowledge, this study constitutes the first large-scale comparative proteomics investigation performed among these largely variable types of egg white samples.

Keywords: Egg white; Gradient-PAGE, HPLC, Native-PAGE, SDS-PAGE

INTRODUCTION

One of the main scientific compasses in many eggs related projects is to use egg white components as a cornerstone in several fields of food and drug industry (Kovacs-Nolan *et al*., 2005, Abu-Ghoush *et al*., 2008, Omana *et al*., 2010). Usually, egg white proteins in birds are rich in essential amino acids, and possess in chickens a valuable nutritional food (Mine, 2008). It contains many individual protein components with high potential for several industrial applications, such as ovalbumin, ovotransferrin, ovomucoid, ovomucin, and lysozyme (Abeyrathne *et al*., 2013). Several parameters that affect egg white were studied, such as heat (Akkouche *et al*., 2012), salt (Kaewmanee *et al*., 2011), pH (Bovskova and Mikova, 2011), and storage (Qiu *et al*., 2012). More than forty different egg white proteins were isolated and identified (Sunwoo & Gujral, 2014). However, almost all the known egg white components were usually separated from chickens

(Awade, 1996, Raikos *et al*., 2006, Guerin-Dubiard *et al*., 2006; D'Ambrosio *et al*., 2008; Mann, 2007; Mann and Mann, 2011). But chickens are not the only birds from which egg white are highly utilized in food and industry. Instead, other egg-white resources were also relied on in this regard, such as quails and ostriches, or even other related species, in several regions around the world. Therefore, the essential importance of chickens being as very abundant and cheap source of egg white proteins aren't satisfied to focus only on this bird without giving a significant highlight on other related species that have a remarkable impact in terms of food and industry. So, relying only on chickens' egg white as the only source for many food and industrial applications may not be sufficient to fulfill all the extended needs of the recent scientific requirements. Despite the magnitudes of the researches conducted in egg whites, there is still a lack of the complete comparative proteomic profile concerning the detailed protein chemical compositions of several egg white varieties. Although, several comparative egg white proteomics studies were performed on several poultry species (Desert *et al*., 2001, Miguel *et al*., 2005, Omana *et al*., 2011, Qiu *et al*., 2012, Wang *et al*., 2012), a complete idea of the whole comparative data to construct a concrete basics on these differences is lacking. Thus, detailed information on the egg white of other species compared with chickens' egg white, weren't abundant enough to build a determined view on the nature and the extent of these differences. Thus, this focus should be broadened to include other bird species. However, it's not rational for the researchers to go further in the various applications of egg white varieties according to their differences without having identified the profile of these differences. Since it is well documented that egg white proteins are one of the best-known bird's proteins (Campell *et al*., 2003), it should be focused on to start evaluating these differences. The profiles of egg white proteins, regarding as the most accessible protein sources, are potentially postulated to occupy valuable roles in protein phenotyping studies of birds. As long as such fluids contain many standarized proteins, many variabilities were possibly available in such a way they could potentially be used in the proteomics diagnosis to differentiate among the types of birds. Undoubtedly, knowing the differences of birds' egg white protein components and their physicochemical properties can enhance the potential applications of birds' egg white in the food industry (Nys and Sauveur, 2004), and therapeutic applications (Narat, 2003), and can also intensify our knowledge's of various biological processes (Wellman-Labadie *et al*., 2008). No large-scale information regarding the main divergence in the whole egg white compositions among different species of bird has been reported. Despite the availability of the previously mentioned studies on the egg whites, the number of data that describe the variability of the egg whites among genera and species still very few in many types of birds. Therefore, the main aim of this study is to highlight the extent of differences among egg white for various birds species. This task can be done by performing a direct screening of the egg white protein to identify the potential molecular categories

of many birds by simple proteomic separation techniques. Hence, it's not the purpose of this investigation to solve the entire chemical composition of the egg white varieties. Rather, its purpose is to determine whether the protein heterogeneity evidence alone can support this suggested diagnostic approach. To our knowledge, this work constitutes a pilot large-scale study that simplifies in-parallel proteomic investigation as its include a direct comparison among more than forty different types of egg white proteins in only of dual gel formats.

MATERIALS AND METHODS

Samples' Collection and Preparation

All samples were collected from different commercial stores and bird keepers from variable regions of middle Euphrates areas in Iraq (Table 1). During a period of about 120 days, 42 eggs from 42 commercially and locally available types of birds, genus or species, were collected, phenotypically classified, and stored in -20°C as whole eggs until processed. In the case of large-sized eggs, the egg white samples were collected from each egg by windowing the sterilized egg shell, while in the case of small-sized eggs, egg white proteins were obtained by cracking the sterilized egg's shell. Then, they were centrifuged for 10 min at 3461 xg at room temperature in a clinical centrifuge (EBA 20, Hettich, Germany). Any spoiled egg component was omitted from this study. All supernatants were kept under -20˚C until processed.

Table 1 A list of the bird resources from which egg white samples were collected

No	species	No	species	No	species	No	species
1	*Columba livia domestica* (Domestic pigeon)	12	*Agapornis fischeri* (Fischer's fischeri)	23	*Coracias garrulous* (European roller)	34	*Agapornis roseicollis* (Rosy-faced lovebird)
2	*Columba livia* (Rock dove)	13	*Melopsittacus undulates* (Budgerigar)	24	*Gallus gallus domesticus* (Chicken)	35	*Alectoris Barbara* (Barbary partridge)
3	*Streptopelia semitorquata* (Red eye dove)	14	*Rollulus rouloul* (Green wood quail)	25	*Agapornis personatus* (Yellow-collared lovebird)	36	*Charadrius dubius* (Little ringed plover)
4	*Streptopelia tranquebarica* (Red turtle dove)	15	*Ammoperdix griseogularis* (See-see Partridge)	26	*Agapornis nigrigenis* (Black-cheeked lovebird)	37	*Galerida crista* (Crested lark)
5	*Columba palambus* (Common wood pigeon)	16	*Ammoperdix griseogularis* (See-see partridge)	27	*Ammoperdix heyi* (Sand partridge)	38	*Nymphicus hollandicus* (Cockatiel)
6	*Streptopelia roseogrisea*	17	*Carduelis carduelis*	28	*Padda oryzivora* (Java sparrow)	39	*Passer domesticus* (House sparrow)

	(African collared dove)		(European goldfinch)				
7	*Streptopelia bitorquata* (Island collared dove)	18	*Falco peregrinus* (Peregrine falcon)	29	*Streptopelia turtur* (European turtle dove)	40	*Treron phoenicoptera* (Yellow-footed green pigeon)
8	*Streptopelia tranquebarica* (Red turtle dove)	19	*Tadorna tadorna* (Common Shelduck)	30	*Agapornis fischeri* (Fischer's lovebird)	41	*Francolinus francolinus* (Black francolin)
9	*Streptopelia decaocto* (Eurasian collared dove)	20	*Alopochen aegyptiacus* (Egyptian goose)	31	*Gallus domesticus* (Faverolles chicken)	42	*Sturnus vulgari* (Common starling)
10	*Meleagris gallopavo* (Domesticated turkey)	21	*Anser anser rubrirostris* (Iraqi goose)	*32*	*Coturnix Coturnix* (Common quail)		
11	*Coturnix adansonii* (African blue quail)	22	*Anas Platyrhynchos* (Domestic duck)	33	Glareola pratincola (Collared pratincole)		

Separation of Egg White Samples by Discontinuous SDS-PAGE

The supernatants were diluted (1:1) in the denaturing-loading buffer (0.5M Tris—HCl, pH 6.8; 4% SDS; 20% glycerol; 10% β-mercaptoethanol and 5% bromophenol blue), and then heated for 3 min at 95°C in a water bath (Memmert, Schwabach, Germany). Each sample was separated by gel electrophoresis on 10% mini vertical gel format, gel size (W×L) cm: 10×10, and gel thickness: 1 mm (Model OmniPAGE, Cleave Scientific – UK), and midi vertical gel format, gel size (W×L) cm: 12×14.5, and gel thickness: 1 mm (Model JY-SCZ9, Junyi-Dongfang Electrophoresis Equipment – China). The discontinuous Laemmli (SDS-PAGE) method was applied (Laemmli, 1970) with minor modifications. For mini gel format, electrophoresis of egg white proteins was performed using 10% separating gel buffer [10% of 30:0.8% acrylamide/bisacrylamide, 1.5M tris-Cl pH8.8, 0.4% (w/v) SDS], and 6% stacking gel buffer [6% of 30:0.8% acrylamide/bisacrylamide, 1M tris-HCl pH6.8, 0.4% (w/v) SDS]. For midi gel format, the concentration of separating gel buffer was changed into 12%. From 9 µg into 15 µg of samples loaded were by mixing 1:1 V/V with sample denaturing-loading buffer (0.5M Tris—HCl, pH 6.8; 4% SDS; 20% glycerol; 10% β-mercaptoethanol and 5% bromophenol blue). Molecular weight prestained standards were also routinely loaded (Bioneer Cat # D-2010). Loaded samples were electrophoresed in 1X of running buffer [25 mM Tris pH 8.3, 250 mM glycine, 0.1% (w/v) SDS] in a vertical electrophoresis tank at 120V and 30 mA for mini gel formats, and 200 V and 85 mA for midi gel formats. Electrophoresis was performed at constant

parameters until the tracking dye reached the end of the gel. Gels were stained with Coomassie blue (Candiano *et al.*, 2004).

Separation of Egg White Samples by Gradient SDS-PAGE

The supernatants were diluted (1:1) in the denaturing-loading buffer, and then heated at 95°C in a water bath for 3 min. Each sample was separated by gel electrophoresis in 4 – 10% the midi vertical gel format. The gradient method of Domingo was applied (Domingo, 1990), with some modifications. Briefly, two solutions were prepared in the casting of the 4 – 10% gradient gel in midi format gels. Solution A (or heavy solution), which includes 10% acrylamide (2.7 ml acrylamide solution, 3.28 ml D.W., 2 ml separating gel buffer, 1.2 g sucrose, 20 µl freshly prepared ammonium persulfate, and 10 µl freshly added TEMED) was prepared. Solution B (or light solution), which includes 4% acrylamide (1.06 ml acrylamide solution, 4.8 ml D.W., 2 ml separating gel buffer, 20 µl freshly prepared ammonium persulfate, and 10 µl freshly added TEMED) was prepared. The total volume of the light and heavy solution is 15 ml, which is sufficient to prepare a gradient gel in a 50 ml capacity disposable syringe. Then, 5% stacking gel [6% of 30:0.8% acrylamide/bisacrylamide, 1M tris-HCl pH6.8, 0.4% (w/v) SDS] was applied above the gradient separating gel. From 9 µg into 15 µg of samples loaded were by mixing 1:1 V/V with sample loading buffer. Molecular weight prestained standards were also routinely loaded (Bioneer Cat # D-2010). Loaded samples were electrophoresed in 1X of running buffer in a vertical electrophoresis tank at 180V and 85 mA, for midi gel formats. Electrophoresis was performed at constant parameters until the tracking dye reached the end of the gel. Gels were stained with Coomassie blue.

Separation of Egg White Samples by Native-PAGE

The same samples preparative procedure mentioned in SDS-PAGE were used. The supernatants were diluted (1:1) in non-denaturing loading buffer (0.5M Tris—HCl, pH 6.8; 4% SDS; 20% glycerol; and 5% bromophenol blue). Each sample was separated by gel electrophoresis on 10% midi gel format. The discontinuous Native-PAGE method was applied (Arndt *et al*., 2012). Electrophoresis of egg white proteins was performed using 10% separating gel buffer [10% of 30:0.8% acrylamide/bis acrylamide, 1.5M tris-Cl pH8.8], and 6% stacking gel buffer [6% of 30:0.8% acrylamide/bisacrylamide, 1M tris-HCl pH6.8]. From 7 µg into 13 µg of samples loaded were by mixing 1:1 V/V with sample loading buffer. Four molecular weight standard proteins were also routinely loaded (14 kd of lysozyme, 31 kd of carbonic anhydrase, 45 kd of ovalbumin, 66 kd of bovine serum albumin, 97 kd of phosphorylase B). Loaded samples were electrophoresed in 1X of running buffer [25 mM Tris pH 8.3, 250 mM glycine] in a vertical electrophoresis tank at 120V

and 30 mA. Electrophoresis was performed at constant parameters until the tracking dye reached the end of the gel. Gels were stained with Coomassie blue.

Separation of Egg White Samples by Cellulose Acetate

Cellulose acetate electrophoresis of egg white was performed according to Keren method (Keren, 2003). CellasGEL 250µm (2.5 x 7 cm) strips were used in these experiments (Cleaver Scientific, Warwickshire, UK). The strips were soaked with agitation for 30 min at room temperature in barbital buffer (Tris Hippurate 0.05 M, pH 8.8, Barbital tris 0.05M). The strips were briefly blotted and immediately spotted with 2 µl of each egg white sample. Electrophoresis was performed by CSL-CELLAS device (Cleaver scientific, Warwickshire – UK) at 200 volts for 35 min at room temperature in barbital buffer. A standard bovine serum albumin fraction V was used as a size marker (BioLabs, London W1W 6DB, UK). Following electrophoresis, the strips were then stained and fixed by immersion in a staining solution [1 g ponceau S, 37.5 g trichloro-acetic acid, 37.5 g sulfosalicylic acid in 500 ml water (w/v)] for 10 min. Then, destaining was performed by washing for several times with gentle agitation in a destaining solution (10 % ethanol, 5 % glacial acetic acid). The strips were dried at room temperature and imaged by a digital camera (Sony – China). The generated images were analyzed by CS analyzer software (ATTO, Yushima, Bunkyo-ku, Japan).

Separation of Egg White proteins by RP-HPLC

RP-HPLC separations were performed according to the method of Miguel (Miguel *et al*., 2005), with some modifications. The egg white proteins were separated by HPLC system equipped with a UV-Visible detector (Knauer advanced scientific instruments, Berlin, Germany). System control and Data acquisition were performed by Clarity chromatography station software (DataApex, Prague, Czech Republic). The analysis was carried with a Discovery® BIO Wide Pore C18 column, with 4.6 X 250 mm, 5 µm (Supelco, Madrid, USA), at ambient temperature. Two solvents were used in the mobile phase of these experiments. Solvent A was 0.1% (v/v) trifluoroacetic acid (TFA) in HPLC-grade water, and solvent B was 0.1% (v/v) TFA in HPLC-grade acetonitrile. Elution was performed at room temperature, with a flow rate of 0.8 ml/min and with a linear gradient from 2 to 65% of solvent B for 60 min then to 75% of solvent B in 90 min. Absorbance was monitored at 214 nm. Before the injection, samples were filtered through 0.45-mm filters (Millipore Corporation, Bedford, MA, USA).

RESULTS AND DISCUSSION

In the present study, the macromolecular components of egg whites were studied by directly submitting egg white components into variable techniques for polyacrylamide gel-based and cellulose acetate based electrophoresis, and RP-HPLC. Irrespective to all the accumulated data, a direct comparison of the effect of genera and species of birds classification on the main components of its egg white profile varieties was very few highlighted. Particularly, the whole egg white proteins of chickens, quails, and ducks have been studied widely (Mann and Mann, 2011; Hu *et al*., 2016; Miguel *et al*., 2005). However, the manuscripts that described the variability of proteins among the egg white from different species of birds were not performed on a large number to build an initial screening data to identify the nature of these differences. In this study, several routinely used electrophoretic techniques, such as denaturing, non-denaturing, and gradient PAGE, were implemented to compare between the benefits and limitations of each one in the accurate discrimination amongst the analyzed egg-white samples. In addition, several routinely used non-electrophoretic experiments were performed to collectively monitor the differences of the whole egg white profile. So, instead of using the commonly used DNA-based diagnostic tools in birds (Pereira *et al*., 2008), several attempts were carried out to use proteomics identifications alternatively. Although the genomic diagnosis is highly accurate, the proteomic diagnosis characterizes with a very high dynamic process since its directly correlated with the changeable protein expression levels (Corthals *et al*., 2000, Fey and Larsen, 2001). Therefore, this study provides an assessment of egg white as a dynamic diagnostic marker using several proteomic routine techniques. The utilization of low-cost and basic analysis techniques may broaden the applications of this diagnosis around the world. SDS-PAGE followed by Coomassie blue detection is one of the routinely available techniques that can be invested with low cost and straightforward identification of the egg white proteins. Nevertheless, SDS-PAGE alone, however, is limited in terms of its low ability to resolve proteins of similar molecular masses (Cassiday, 2007). Thus, it should be aided with another electrophoretic technique to overcome its shortcomes in the detection of several unknown protein bands. Therefore, in addition to the submitting egg white samples into variable SDS-PAGE conditions, other techniques were applied, such as Native-PAGE, and cellulose acetate. Because of low reproducibility that originated from batch to batch variability (Magdeldin *et al*., 2014), isoelectric focusing (IEF) wasn't used in this study. Also, the labor-intensive 2D-PAGE wasn't included in this research as it cannot analyze total proteins straightforwardly because the cellular content of egg white protein varieties was very high, and this highly complicates the interpretation of the resolved proteins (Bunai and Yamane, 2005). On the other hand, hydrophobic HPLC was applied to give a further fingerprint about the whole nature of these samples with regard to proteins function and specificity.

SDS-PAGE

Several denaturing electrophoretic conditions in terms of varying gels and sample concentrations to show the most beneficial profile. Several other technical standardizations were made, such as maximizing samples numbers in each gel format to enhance the chances of the correct in-parallel reading, were made. They were optimized as much as possible to provide a direct and simultaneous comparison among a larger number of samples. The small mini gels formats weren't competent enough to provide an accurate in-parallel comparison of the egg white bands. Therefore, larger formats and greater wells numbers were included to load as many samples as possible in one gel format. Thus, the sizes of gels and the number of wells were approximately duplicated. Moreover, each individual concentration of separating gel could precisely describe a certain range of proteins and relatively neglect the other proteins of other molecular weights (Rath *et al*., 2009). Therefore, two different concentrations of gel were used in each case. However, since polyacrylamide gel electrophoresis is very sensitive techniques to any tiny changes in protein profile, two variable concentrations of egg white proteins were applied (Fig. 1). However, relying on MW, many proteins were identified in the literature in many egg white samples (Awade, 1996, Cao, 2005, Sunwoo & Gujral, 2014).

Although the silver staining technique is very sensitive in comparison with Coomassie counterpart (Weiss *et al*., 2009), it was omitted from the staining because of several limiting practical factors, such as the differences of development time may give non-real quantitative density of the proteins bands as several proteins were obscured because of the dark areas that emerged during development (Gromova and Celis, 2006). Also, since the very high sensitivity of silver nitrate stain several non-proteinaceous portions, and thus it was found that this procedure is further complicating the reading of the gel (data not shown). Some of the proteins bands were clearly identified by simple direct comparison with their standards, while other bands were not. This concomitant difficulty of gel reading interpretations could not be resolved without submitting the same samples into further conditions. This difficulty was not easy to be excluded from the research since it was found that several egg white proteins have very close MW (Desert *et al*., 2001).

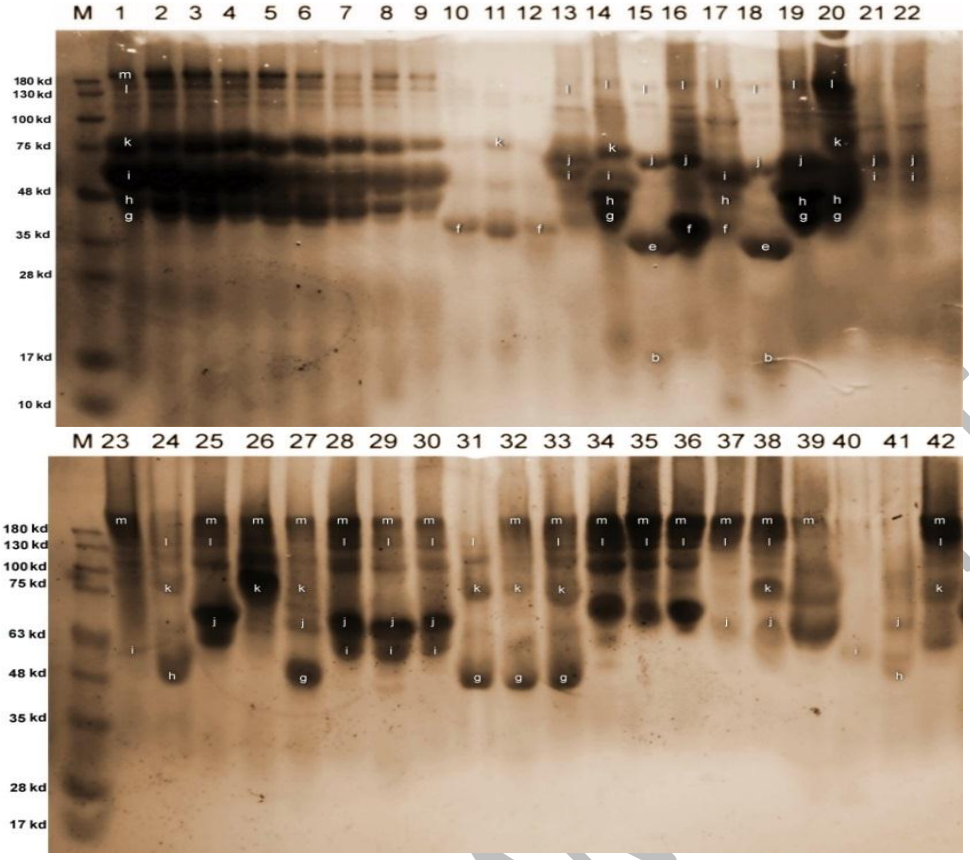

Figure 1 SDS-PAGE of egg white protein samples in 12% midi gel formats. Lane "M" refers to ladder marker. Lanes 1 – 42 refers to variable birds' egg white protein samples. The letters "a" into "m" refers into the egg white resolved proteins

In addition to the limited range of proteins to be resolved on the gel, several extremely high and low molecular weight standards were not easily available for comparison (Hu *et al*., 2016). Nonetheless, several simple electrophoretic migrations in this study were provided fruitful data on distinct resolving power on many egg white based only one-dimensional electrophoresis. Despite the high electrophoretic variability among the egg white samples, a particular pattern of distribution of egg white proteins was observed in some phenotypically related samples. This has obviously been noticed in the first nine samples, that are very closely related to each other in terms of classification. It was found that all the applied electrophoretic conditions of these samples have shown very close biological relationships. This, in turn, indicates the potential validity of these simple electrophoretic conditions to provide an initial diagnostic marker among these samples. On the other hand, interesting differences between the egg white patterns of a species from other distant families were found. However, not only the discrete differences among the isolated and identified egg white proteins identities are known, the differences of their concentrations are known too (Miguel *et al*., 2005). In other words, this result refers to the potential eligibility of this simple one-dimensional SDS-PAGE to give us the extent of phenotypic divergence among birds only through this low cost and rapid tool for screening.

Despite performing several repetitions of the electrophoretic separation, the encountered practical difficulties of these egg white samples were inevitable in many instances, as it is relatively hard to standardize these variable viscosity specimens simultaneously in only one gel format. This is one of the factors that forced us to submit them into variable concentrations of SDS-PAGE and other electrophoretic environments. Another factor is related to this action is the vast gap of protein concentrations that exist among variable egg white protein composition. For instance, ovalbumin, ovotransferrin and ovomucoid represent about 77% of egg white content (Mine *et al*., 1995), while other components never exceeded 1%, such as avidin and flavoprotein (Desert *et al*., 2001). So, to improve detection of proteins in such samples, different amounts of proteins were loaded (Fig. 2). However, several protein bands were unambiguously identified in most of the samples.

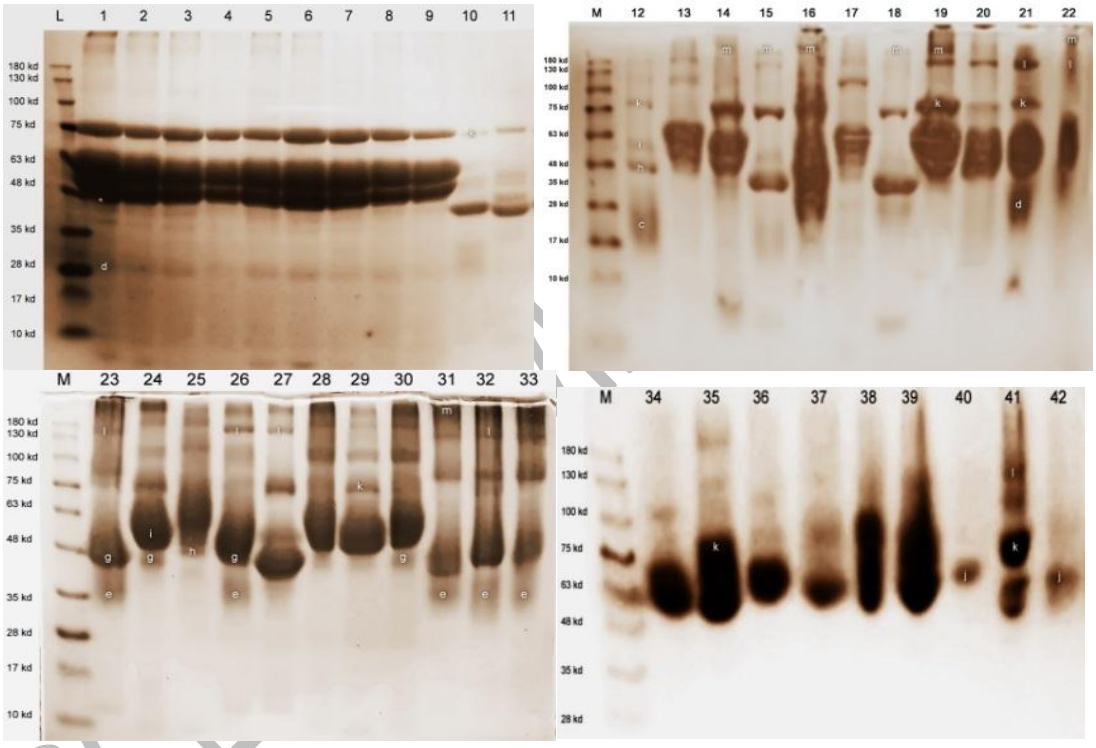

Figure 2 SDS-PAGE of egg white protein samples in 10 % mini gel formats. Lane "M" refers to ladder marker. Lanes 1 – 42 refers to variable birds' egg white protein samples. The letters "a" into "m" refer into the egg white resolved proteins that don't resolve in Fig. 1.

It deserves to note that the most abundant proteins in the studied samples are ovomucin proteins (MW 135 – 150 kd, and 220 – 270 kd) (Alleoni, 2006). While cystatin (MW 13 kd), as in Table 2, was not seen in all samples as it is a minor protein (Abeyrathne *et al*., 2013).

Table 2 The expected observed bands of the birds' egg white samples and their corresponding proteins according to variable PAGE conditions.\

	Description of Known Proteins Bands												
	a Cystatin	**b** Lysozyme	**c** Ovoglycoprote	**d** Ovomucoid,	**e** Ovoflavoprotei	**f** Thiamine binding	**g** Ovalbumin	**h** G_2 Ovoglobulin	**i** Ovoinhibitor	**j** Avidin	**k** Ovotransferrin	**l** Ovomucin	**m** Ovomucin
MW (Kd)	**13**	**14**	**24**	**28**	**32-35**	**38**	**45**	**47**	**54**	**67-68**	**76-78**	**135-150**	**220-270**
No. of samples													
1	-	-	-	+	-	+	+	+	+	+	+	+	+
2	-	-	-	+	-	+	+	+	+	+	+	+	+
3	-	-	-	+	-	+	+	+	+	+	+	+	+
4	-	-	-	+	-	+	+	+	+	+	+	+	+
5	-	-	-	+	-	+	+	+	+	+	+	+	+
6	-	-	-	+	-	+	+	+	+	+	+	+	+
7	-	-	-	+	-	+	+	+	+	+	+	+	+
8	-	-	-	+	-	+	+	+	+	+	+	+	+
9	-	-	-	+	-	+	+	+	+	+	+	+	+
10	-	-	-	-	+	+	+	+	-	-	+	-	-
11	-	-	-	-	+	-	+	-	-	+	+	-	-
12	-	-	+	-	+	+	+	+	+	+	+	-	-
13	-	+	-	+	-	-	+	-	+	+	-	+	-
14	-	-	-	-	-	-	+	+	+	-	+	+	+
15	-	+	-	-	+	+	+	-	-	+	-	+	+
16	-	-	+	-	-	+	-	-	-	+	-	+	+
17	-	-	-	-	-	+	-	+	+	-	-	+	-
18	-	+	-	+	+	-	+	-	-	+	-	+	+
19	-	-	-	-	-	-	+	+	-	+	+	+	+
20	-	+	-	-	+	-	+	+	-		+	+	-
21	-	+	-	+	+	-	-	-	+	+	+	+	-
22	-	+	-	-	+	-	-	-	+	+	-	+	+
23	-	-	-	-	+	-	+	-	+	-	-	+	+
24	-	-	-	-	-	-	+	+	+	-	+	+	-
25	-	-	-	-	+	-	-	+	-	+	-	+	+
26	-	-	-	-	+	-	+	-	-	-	+	+	+
27	-	-	-	-	-	-	+	-	+	+	+	+	+
28	-	-	-	-	+	-	-	-	+	+	-	+	+
29	-	-	-	-	+	-	-	-	+	+	+	+	+
30	-	-	-	-	+	-	+	-	+	+	-	+	+
31	-	-	-	-	+	-	+	-	-	-	+	+	+
32	-	-	-	-	+	-	+	-	+	-	+	+	+
33	-	-	-	-	+	-	+	-	-	-	+	+	+
34	-	-	-	-	+	-	-	-	-	-	-	+	+
35	-	-	-	-	-	-	-	-	+	-	+	+	+
36	-	-	-	-	+	-	-	-	-	-	-	+	+
37	-	-	-	-	-	-	-	-	-	+	-	+	+
38	-	-	-	-	-	-	-	-	-	+	+	+	+

39	-	-	-	-	-	-	-	-	-	-	-	-	+
40	-	-	-	-	-	-	-	-	+	+	-	-	-
41	-	-	-	-	-	-	-	+	-	+	+	+	-
42	-	-	-	+	+	-	-	-	-	+	+	+	+

The main electrophoretic limitation for egg white separation was potentially attributed to the ability of each gel concentration to separate certain MW range of proteins. This, in turn, led to the fact that not all the identities of many other MW bands were not yet known (Table 3).

Table 3 The unknown observed bands of the birds' egg white samples according to variable PAGE conditions

No. of samples	**Description of Unknown Proteins Bands**							
MW (Kd)	**4-6**	**63**	**83-85**	**90-95**	**100-105**	**115-117**	**120-123**	**125-129**
1	+	+	-	-	-	+	-	+
2	+	+	-	-	-	+	-	+
3	+	+	-	-	-	+	-	+
4	+	+	-	-	-	+	-	+
5	+	+	-	-	-	+	-	+
6	+	+	-	-	-	+	-	+
7	+	+	-	-	-	+	-	+
8	+	+	-	-	-	+	-	+
9	+	+	-	-	-	+	-	+
10	-	-	-	-	-	-	-	-
11	-	-	-	-	-	-	-	-
12	-	-	-	-	-	-	-	-
13	-	-	-	-	-	+	-	-
14	-	+	-	-	-	+	-	-
15	-	-	-	-	+	+	-	-
16	-	-	-	-	-	+	-	-
17	-	+	-	-	+	-	-	-
18	-	-	-	-	-	-	-	-
19	-	-	-	+	+	+	-	-
20	-	-	-	-	-	-	-	-
21	-	-	-	+	+	-	-	-
22	-	-	-	+	+	-	-	-
23	-	-	+	-	-	-	+	-
24	-	-	-	-	-	+	-	-
25	-	-	-	-	+	-	-	-
26	-	-	-	-	+	-	-	+
27	-	-	-	-	+	-	-	-
28	-	-	-	-	+	-	-	-
29	-	-	-	-	+	-	-	-
30	-	-	-	-	+	-	-	-
31	-	-	-	-	+	-	-	-
32	-	-	-	-	+	-	-	-
33	-	-	-	-	+	-	-	-
34	-	-	-	-	+	-	-	-
35	-	-	-	-	+	-	-	-

36	-	-	-	-	+	-	-	-
37	-	-	-	-	+	-	-	-
38	-	-	-	-	-	-	-	-
39	-	-	-	+	-	-	-	-
40	-	-	-	-	-	-	-	-
41	-	-	-	-	-	-	-	-
42	-	+	-	-	-	+	-	-

This, however, was minimized by submitting egg white samples into variable PAGE conditions. Even though, these variable electrophoretic conditions were applied, they still have inevitable limitations in terms of lack of discrimination between the variable forms of proteins because of several reasons, such as glycosylation (Jay *et al*., 1990), and phosphorylation (Li *et al*., 2003), or into the splitting of some proteins into smaller subunits in the reducing conditions (Hoppe, 2010). Although these techniques identified many proteins according to their MW differences on the gel, it's not known whether these differences are attributed into the various posttranslational modifications that might be followed by some of these proteins in their three-dimensional structure, amino acids residues, in their backbones, or into discrete differences in their amino acid sequences. However, the majority of egg white samples are polymorphic in nature (Guearin-Dubiard *et al*., 2006), and this adds more complication in their direct comparative visualization. Its deserve to note that the presence of certain physical barriers in the egg white samples stands against the electrophoretic separation of several experiments of the whole egg white samples. However, the electrophoretic experiments were repeated several times since it is not easy going sometimes to directly separate them because of the obviously noticed steric resistance that induced by the carbohydrate moieties (Desert *et al*., 2001). This fact may be explained by the high viscosity originated from the presence of ovalbumin (Alleoni, 2006). Moreover, other difficulties are noticed when glycoproteins migrate unpredictably in SDS-electrophoresis because the sugar moieties do not bind SDS (Hames, 1998). Hence, if the purpose of this study is to perform an in-depth analysis of these egg white samples, 2-dimensional electrophoresis and MALTI-TOF analysis are prerequisites in this aspect (Hu *et al*., 2016). Gradient gel electrophoresis can allow a greater range of separation if both large and small proteins MW need to be resolved simultaneously in only one gel format (Brunelle & Green, 2014). However, several proteins, such as ovalbumin (sample No. 13), ovoflavoprotein (samples No. 25, 28, 29, 30, 36, and 42), and ovomucoid (sample No. 42) were not resolved in discontinuous SDS-PAGE (Fig. 3).

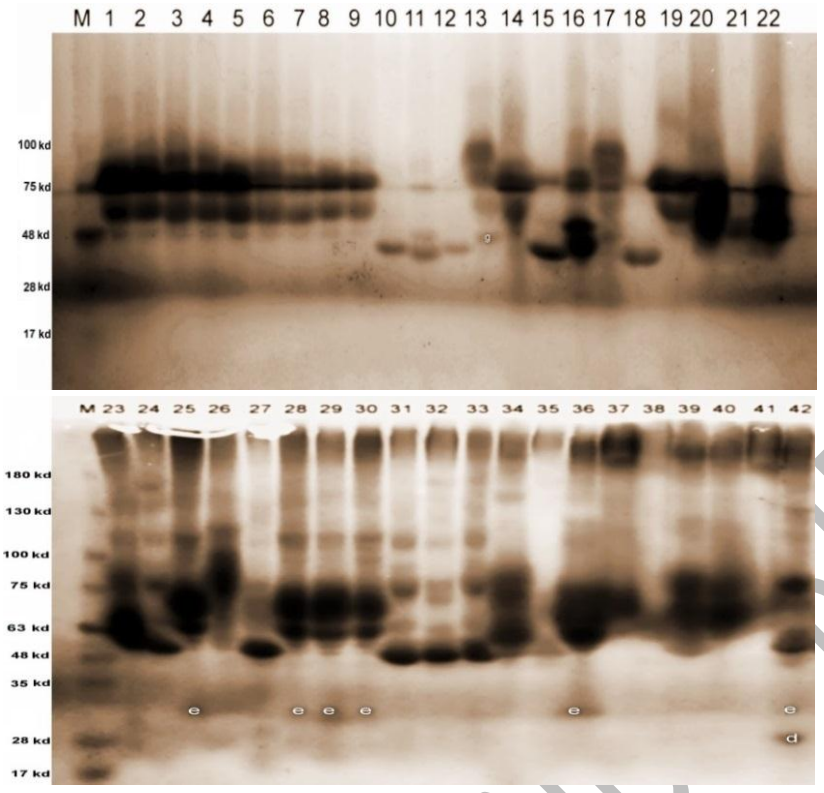

Figure 3 Gradient-PAGE of egg white protein samples. Lane "M" refers to ladder marker. Lanes 1 – 42 refers to variable birds' egg white protein samples. The letters "a" into "m" refers into the egg white resolved proteins that don't resolve in Fig. 1 and Fig. 2.

Native-PAGE

Although, SDS-PAGE is the most popular method due to their availability, reproducibility, and ease of use, the situation for complicated proteins differ in terms of having more reaction sites as its seen in these variable egg white samples, so, SDS-PAGE alone may not offer the best resolution required (Zheng *et al*., 2007).

To achieve a comprehensive understanding of cellular proteins, the limitations of SDS-PAGE should be overcome by adding other methods such as Native-PAGE. Thus, it is interesting in this study to directly submit these variable egg white samples into Native PAGE as many proteins lose their natural conformations in the commonly used SDS-PAGE deliberately created denaturing conditions, and because of the reducing conditions, they tend to behave in a manner that does not resemble their habit in nature (Nowakowski *et al*., 2014). Though native-PAGE is not commonly used in the usual diagnosis of many protein samples (Gallagher, 1999), it is mandatory to expose these variable samples into the non-denaturing conditions in order to take a snapshot on many unknown samples that are not easily identified in SDS-PAGE conditions. As it was expected, another unique pattern was observed. But, irrespective of this unique resolution, the same pattern of distributions of almost all samples was observed (Fig. 4). On the other hand, it is relatively difficult to calculate a lot of proteins MW according to their native separation. The paucity of any previous

Native-PAGE is the main reason for this difficulty. Rather, the monitoring of the natural behavior of many proteins that have relatively close MW, may increase the difficulty of this task. However, several proteins, such as ovoglycoprotein (sample No. 16), ovomucoid (samples No. 13 and 18), ovoflavoprotein (samples No. 10, 11, 12, 20, 21, and 22), thiamine binding protein (sample No. 15), ovalbumin (samples No. 10, 11, 12, 15, and 18), G3 ovoglobulin (sample No. 10), ovoinhibitor (samples No. 27, 32, and 53), and avidin (samples No. 10, 11, and 12) that were not resolved in SDS-PAGE were identified using Native-PAGE.

In this study, through both denaturing and nondenaturing electrophoretic techniques, multiple common bands were resolved in most of the samples, such as 32 – 35, 45, 47, 54, 67 – 68, 76 – 78, 135 – 150, and 220 – 270 KDa, which represent ovoflavoprotein, ovalbumin, G_3 ovoglobulin, ovoinhibitor, avidin, ovotransferrin, and ovomucin I and ovomucin II, respectively. Moreover, as it was mentioned previously, all these proteins were obviously identified. So that, in the electrophoretic portion of this study, several proteins were localized with certainty, which is the following: ovoglycoprotein (MW 24kd), ovomucoid (MW 28kd), ovoflavoprotein (MW 32-35kd), thiamine binding protein (MW 38kd), ovalbumin (MW 45kd), G3 ovoglobulin (MW 47kd), ovoinhibitor (MW 54kd), avidin (MW 67-68kd), ovotransferrin (MW 76-78kd), and ovomucins (MW 135-150 and 220-270kd). However, many bands still interestingly unknown and remain to be recognized individually. On the other hand, in addition to the collectively high resolving power of these several one directional electrophoretic techniques in the in-parallel detection of many of these protein types, it might be possible for some of these techniques to give us a semi-quantitative indication of the intensity of each particular protein per lane. For instance, it was found in this study that the overall ovalbumin concentration occupied the most noticeable quantity of the separated proteins. This agrees with the literature, which constitutes 54% of the total proteins (Stadelman and Cotterill, 2001), while the overall concentration of ovomucoid bands occupied very low quantity of the resolved egg white proteins. However, ovomucoid is a highly glycoslyated protein, so its actual MW is characterized by its changeability in electrophoresis (Kovacs-Nolan *et al*., 2000). However, ovomucoid concentration does not exceed 11% of the total egg white proteins (Caubet and Wang, 2011). This, in turn, optimizes our view in many diagnostic aspects.

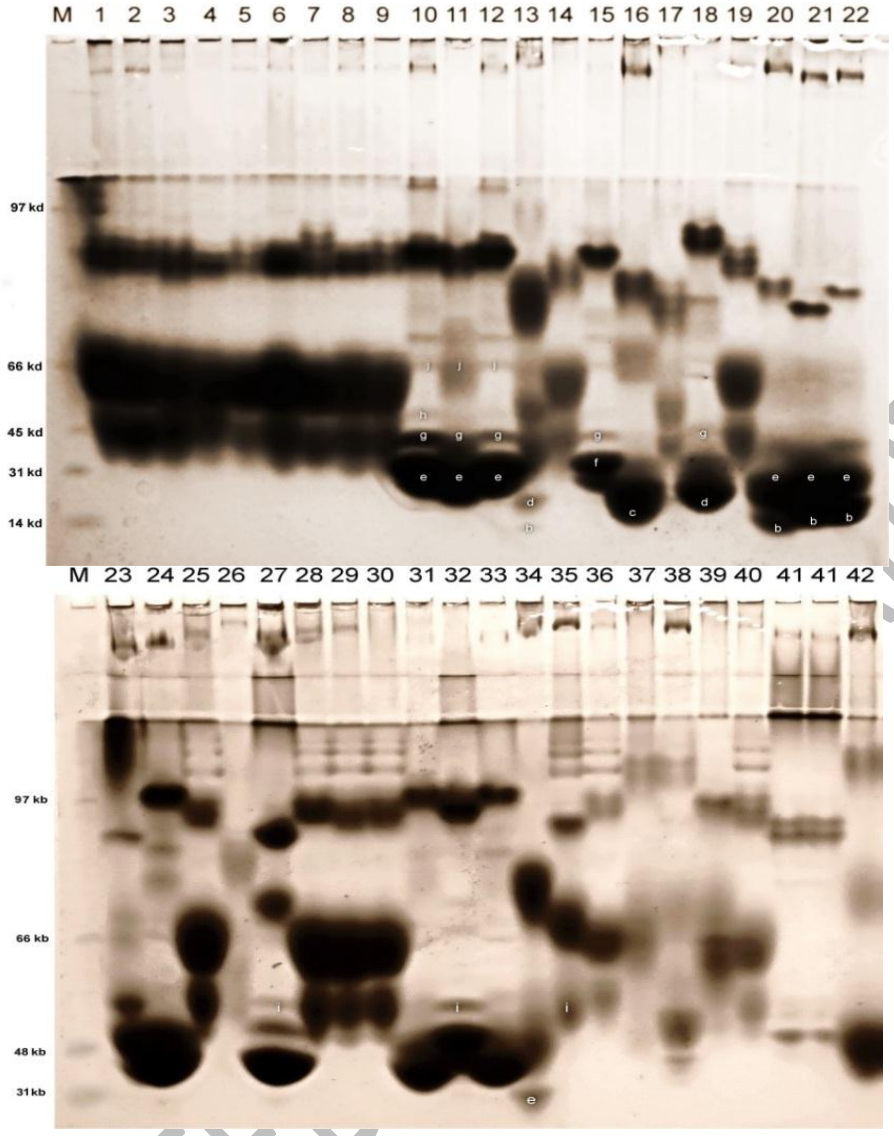

Figure 4 Native Polyacrylamide Gel (Native-PAGE) electrophoresis of high concentrations of egg white protein samples. Lane "M" refers to ladder marker. Lanes 23 – 42 refers to variable birds' egg white protein samples. The letters "a" into "m" refers into the egg white resolved proteins that don't resolve in Fig. 1, Fig. 2, Fig. 3.

Cellulose Acetate Electrophoresis

To further sustain our screening impression of the natural behavior of the egg white samples, the whole egg white were submitted to the cellulose acetate membranes in a non-biased sequential manner (Fig. 5). Despite the observed low resolution of cellulose acetate method, it provided us with interesting information about the charges of egg white proteins. Interestingly, six egg white samples were demonstrated one positively charged bands (sample No. 9, 11, 18, 24, 27, and 33). This naturally existing positively charged proteins or emulsifiers weren't abundantly available in food in their natural biological fluids (Decker, 1998). In addition to its relatively low resolving power that observed from its reduced number of the observed band (Table 4), it is being

reasonable to say that cellulose acetate results weren't categorically correspondingly with the phenotypic classificational differences of the electrophoresed egg white samples.

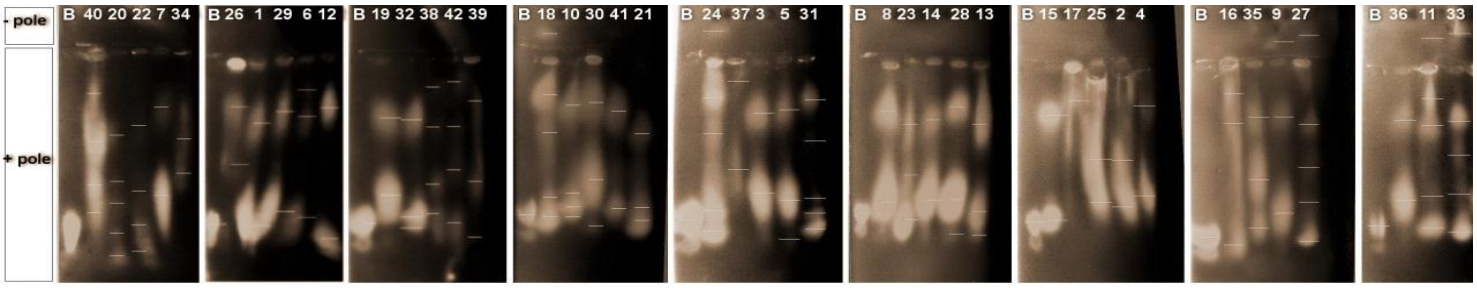

Figure 5 Cellulose acetate gel electrophoresis of egg white proteins samples. Lane "B" refers to bovine serum albumin fraction V marker. Lanes 1 – 42 refers to variable birds' egg white protein samples. The color is inverted into black and white to get better resolution.

RP-HPLC

Although discontinuous and gradient gel electrophoresis systems have been described for egg white separation, the use of RP-HPLC may also be helpful with the intention of monitoring the resolving power, as well as assessing the degree of proteins specificity with regard to their functions. Relatively, similar patterns of the resolution were observed in almost all samples (Fig. 6). This potentially indicates a similar functional specificity that adopted from most of the egg whites proteins within the eggs' environment. However, the typical high resolution of RP-HPLC is significantly reduced in resolving structurally similar components from a complex mixture (Mitulović, 2015). In such cases, a sufficient time is needed to separate the great number of peaks from each other. This has extended run time for these 42 samples in more than 60 hours. Therefore, the runtime was extended into 90 min. Other limitations were very known in these experiments. Such as, the HPLC could not usually be performed for more than one sample at a time.

Table 4 A sum up of the behavior of each type of egg white in cellulose acetate electrophoresis

No. of samples	Positive bands	The relative distance of the negative bands with respect to bovine albumin		
1	-	0.59	1.00	
2	-	0.72	0.93	
3	-	0.52	0.88	
4	-	0.74	0.88	
5	-	0.50	0.91	1.09
6	-	0.4	0.57	0.97

		6				
7	-	0.38	0.83			
8	-	0.54	0.95			
9	+	0.51	0.90			
10	-	0.54	0.91	0.97	1.01	
11	+	0.44	0.56	0.87	1.01	
12	-	0.53	1.06			
13	-	0.60	0.99	1.10		
14	-	0.58	0.94			
15	-	0.51	0.99			
16	-	0.41	0.54	1.03		
17	-	0.44				
18	+	0.50	0.66	0.93	1.01	
19	-	0.35	0.83			
20	-	0.52	0.76	0.87	1.02	1.14
21	-	0.66	0.97	1.03		
22	-	0.47	0.81	0.98	1.07	
23	-	0.60	0.83	1.03		
24	+	0.45	0.61	0.96	1.05	
25	-	0.71	0.91			
26	-	0.53	0.76			
27	+	0.54	0.72	0.86	1.02	
28	-	0.52	0.94	1.09		
29	-	0.55	0.95			
30	-	0.53	0.85	1.05		
31	-	0.46	0.64	0.90	0.97	1.04

32	-	0.36	0.96	1.03	
33	+	0.53	0.69	0.86	1.03
34	-	0.40	0.54	0.71	
35	-	0.51	0.77	0.96	
36	-	0.54	0.89		
37	-	0.38	0.77		
38	-	0.16	0.41	0.76	0.94
39	-	0.24	0.74	1.09	
40	-	0.31	0.56	0.78	0.91
41	-	0.57	0.97		
42	-	0.13	0.40	067	1.00

Consequently, RP-HPLC is limited in this direct comparative diagnostics scope even when it's being used depending on the size exclusion property. In contrast to the electrophoretic techniques that have given the high diversity of the electrophoresed proteins, RP-HPLC doesn't provide such high diversity. However, in this study, the electrophoretic separation was provided interesting superiority compared with RP-HPLC. Concerning the study of variation, RP-HPLC may be failing to give the desired categorizing information about the actual heterogeneity of egg white varieties. In addition, as in some cases in Fig. 6, the HPLC peaks may be broad and overlapping due to the heterogeneity of the egg white samples. This might be attributed to the complexity of the adsorption mechanism of protein aggregates in hydrophobic interaction chromatography that was not fully understood (Mahn, 2012). Nevertheless, through utilizing RP-HPLC, a noticeable conservative nature of almost all studied proteins was observed. The predominant characteristic in egg white could be attributed to the presence of egg white with similar functions, as shown with the similar hydrophobicity peaks (Fig. 6).

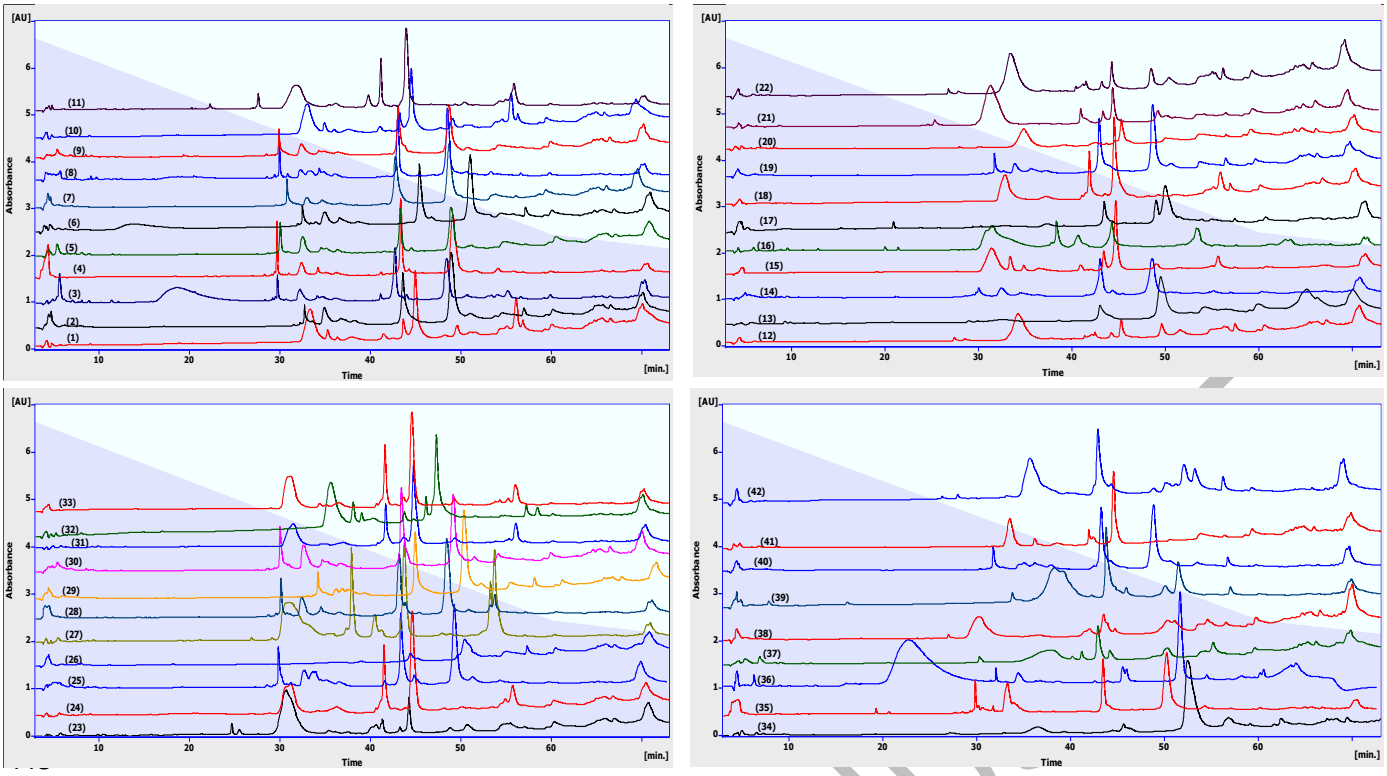

Figure 6 Reverse phase high performance liquid chromatography (HPLC) for egg white proteins. The number of each lane is indicated in each chromatogram.

However, in addition to very long time run of all of these egg white samples because of the inability of HPLC to provide a simultaneous run of all samples, it has limited ability to verify samples on the basis of their hydrophobicity. Thus, the closely related nature of egg white proteins was elucidated through RP-HPLC. Nonetheless, some samples exerted unique peaks in certain portions of elution, such as sample No. 3 and No. 29. Moreover, RP-HPLC provides an initial clue through the similar peaks despite all the egg white high diversity obtained from other techniques, these differences were not attributed to their functions. Instead, other potential factors were involved in this interpretation, such as phenotypic classificational differences. In other words, RP-HPLC results provide an additional indicator for the possibility of using egg white as initial diagnostic tools on the basis of their bird phenotypic classification. Therefore, it might be possible to describe these variabilities as "species related" instead of being "function related" differences.

In contrast to our study, other studies indicated that the differences in the phenotypically variable eggs are not related to chemical compositions; instead, the concentrations of its individual proteins are exposed to such variation among the varieties of eggs (Wang *et al.*, 2012). However, in this study, both egg whites related heterogeneity was obviously observed; the qualitative in which characteristic protein alterations were noticed, and quantitative in which discrete variations of egg white proteins were noticed too.

CONCLUSION

In conclusion, obvious differences between egg white proteins among the different bird types were noticed electrophoretically. In this study, the results indicated that both Native and SDS-PAGE method produced better resolution and also they have the potential to be developed as egg white diagnostic methods. Therefore, this may give a possibility to involve them to provide an initial diagnostic marker to differentiate among different species of birds through their egg white. Irrespective to some additional data that RP-HPLC has provided, it does not give a satisfactory reliability to diagnose these bands. The electrophoretic differences might pave the way for more rapid screening studies by further optimizing the several conditions in SDS-PAGE. This performance can be done by minimizing the gel-based efforts into the extremely acceptable level to provide a more reproducible diagnostic tool to differentiate among various types of egg white of birds.

ACKNOWLEDGMENTS

Many thanks to Zahraa Mohammed, Hibah Salman, Mohammed Hamzah, Ayaat Maan, and Karrar Salam (dept. of animal production/college of agriculture/Al-Qasim Green University) for providing most of the birds' egg white samples for this research.

REFERENCES

Abeyrathne ED, Lee HY, Ahn DU, (2013). Egg white proteins and their potential use in food processing or as nutraceutical and pharmaceutical agents—A review. Poultry Science 92: 3292–3299.

Abu-Ghoush M, Herald, TJ, Aramouni FM, (2010). Comparative study if egg white protein and egg alternatives used in an angel food cake system. Journal of Food Processing and Preservation 34: 411–425.

Akkouche Z, Aissat L, Madani K, (2012). Effect of Heat on Egg White Proteins. ISALS, 10-12: 407 – 413.

Alleoni, AC (2006). Albumin protein and functional properties of gelation and foaming. Sci. Agric. 63 (3): 291-298.

Arndt C, Koristka S, Bartsch H, Bachmann M, (2012). Native polyacrylamide gels. Methods Mol Biol. 869:49-53.

Awade AC, (1996). On hen egg fractionation: applications of liquid chromatography to the isolation and the purification of hen egg white and egg yolk proteins. Z Lebensm Unters Forsch 202:1-14.

Bovskova H, Mikova K, (2011). Factors Influencing Egg White Foam Quality. Czech J. Food Sci. 29 (4): 322–327.

Brunelle JL, Green R, (2014). One-dimensional SDS-Polyacrylamide Gel Electrophoresis (1D SDS-PAGE). Methods Enzymol. 541:151-9.

Bunai K, Yamane K, (2005). Effectiveness and limitation of two-dimensional gel electrophoresis in bacterial membrane protein proteomics and perspectives J. Chromatogr. B 815: 227–236.

Campbell L, Raikos V, Euston SR, (2003). Modification of functional properties of egg white proteins. A review. Nahrung/Food, 47 (6): 369–376.

Candiano G, Bruschi M, Musante L, Santucci L, Ghiggeri G, Carnemolla B, Orecchia P, Zandi L, Righetti P, (2004). Blue silver: A very sensitive colloidal Coomassie G-250 staining for proteome analysis. Electrophoresis, 25, 1327–1333.

Cao L, (2005). Protein Separation with Ion-exchange Membrane Chromatography. Thesis /Faculty Of the Worcester Polytechnic Institute / USA.

Cassiday LT, (2007). Focusing on improved SDS-PAGE separations. Anal Chem. 79(3): 797.

Caubet JC, Julie Wang J, (2011). Current understanding of egg allergy. Pediatr Clin North Am. 1, 58(2): 427–443.

Corthals GL, Wasinger VC, Hochstrasser DF, Sanchez JC, (2000). The dynamic range of protein expression: a challenge for proteomic research. Electrophoresis, 21: 1104-15.

D'Ambrosio C, Arena S, Scaloni A, Guerrier L, Boschetti E, Mendieta ME, Citterio A, and Righetti PG, (2008). Exploring the chicken egg white proteome with combinatorial peptide ligand libraries. J. Proteome. Res. 7:3461–3474.

Decker EA, (1998). Antioxidant Mechanisms. Food Sci. Technol. 88: 397-421.

Desert C, Gue´rin-Dubiard C, Nau F, Jan G, Val F, Mallard J, (2001). Comparison of Different Electrophoretic Separations of Hen Egg White Proteins. J. Agric. Food Chem. 49: 4553-4561

Domingo A, (1990). Exponential Gradient Maker Using a Disposable Syringe. Anal. Biochem. 189: 88 – 89.

Fey SJ, Larsen PM, (2001). 2D or not to 2D. Current Opinion in Chemical Biology, 5: 26–33.

Gallagher SR, (1999). One-dimensional electrophoresis using nondenaturing conditions. Curr. Protoc. Mol.Biol. 47:10.2B.1-10.2B.11.

Gromova I, Celis JE, (2006). Protein Detection in Gels by Silver Staining: A Procedure Compatible with Mass-Spectrometry. From: Cell Biology: A Laboratory Handbook. 3rd Edition. v.4 Eds: Celis, J.E., Carter N., Hunter T., Simons K., Small J.V. and Shotton D. Elsevier. Academic Press.

Guérin-Dubiard C, Pasco M, Mollé D, Désert C, Croguennec T, Nau F, (2006). Proteomic Analysis of Hen Egg White. J. Agric. Food Chem. 54: 3901-3910.

Hames BD, (ed), Gel Electrophoresis of Proteins: A Practical Approach, 3rd edn, Oxford University Press, Oxford, New York (1998).

Hoppe A, (2010). Examination of egg white proteins and effects of high pressure on select physical and functional properties. Dissertations & Theses in Food Science and Technology, University of Nebraska – Lincoln.

Hu S, Qiu N, Liu Y, Zhao H, Gao D, Song R, Ma M, (2016). Identification and comparative proteomic study of quail and duck egg white protein using 2-dimensional gel electrophoresis and matrix-assisted laser desorption/ionization time-of-flight tandem mass spectrometry analysis. Poultry Science 0:1–8.

Jay GD, Culp DJ, Jahnke MR, (1990). Silver staining of extensively glycosylated proteins on sodium dodecyl sulfate-polyacrylamide gels: enhancement by carbohydrate-binding dyes. Anal. Biochem. 185: 324-330.

552 Kaewmanee T, Benjakul S, Visessanguan W, (2011). Effect of NaCl on thermal aggregation of egg
553 white proteins from duck egg. Food Chemistry 125: 706–712

554 Keren DF, (2003). Protein Electrophoresis in Clinical Diagnosis . Hodder Arnold, a member of the
555 Hodder Headline Group, 338 Euston Road, London NW1 3BH.

556 Kovacs-Nolan J, Phillips M, Mine Y, (2005). Advances in the value of eggs and egg components
557 for human health. J. Agric. Food Chem. 53:8421–8431.

558 Laemmli UK, (1970). Cleavage of structural proteins during the assembly of the head of
559 bacteriophage T4. Nature 227: 680-685.

560 Li CP, Salvdor AS, Ibrahim HR, Sugimoto Y, Aoki T, (2003). Phosphorylation of Egg White
561 Proteins by Dry-Heating in the Presence of Phosphate. J. Agric. Food Chem. 51: 6808-6815.

562 Magdeldin S, Enany S, Yoshida Y, Xu B, Zhang Y, Zureena Z, Lokamani I, Yaoita E,Yamamoto T,
563 (2014). Basics and recent advances of two dimensional- polyacrylamide gel electrophoresis.
564 Clinical Proteomics 11:16.

565 Mahn A, (2012). Hydrophobic Interaction Chromatography: Fundamentals and Applications in
566 Biomedical Engineering, Biomedical Science, Engineering and Technology, Prof. Dhanjoo
567 N. Ghista (Ed.), ISBN: 978-953-307-471-9.

568 Mann K, (2007). The chicken egg white proteome. Proteomics. 7:3558–3568.

569 Mann K, Mann M, (2011). In-depth analysis of the chicken egg white proteome using an LTQ
570 Orbitrap Velos. Proteome Science 9:7.

571 Miguel M, Manso MA, Lopez-Fandino R, Ramos, M, (2005). Comparative study of egg white
572 proteins from different species. Eur Food Res Technol 221:542–546.

573 Mine Y, (1995). Recent advances in the understanding of egg-white protein functionality. Trends in
574 Food Science & Technology, 6 (7), 225-232.

575 Mine Y, (2008). Egg Bioscience and Biotechnology. Department of Food Science. University
576 Guelph of. Willy - Interscience. ISBN: 978-0-470-03998-4.

577 Mitulović G, (2015). New HPLC Techniques for Proteomics Analysis: A Short Overview of Latest
578 Developments. Journal of Liquid Chromatography & Related Technologies, 38: 390–403.

579 Narat M, (2003). Production of Antibodies in Chickens. Food Technol. Biotechnol. 41 (3) 259–267.

580 Nowakowski AB, Wobig WJ, Petering DH, (2014). Native SDS-PAGE: High Resolution
581 Electrophoretic Separation of Proteins With Retention of Native Properties Including Bound
582 Metal Ions. Metallomics 6 (5): 1068–1078.

583 Nys Y, Sauveur B, (2004). Nutritional Value of eggs. INRA Prod. Anim. 17 (5): 385-393.

584 Omana DA, Wang J, Wu J, (2010). Co-extraction of egg white proteins using ion-exchange
585 chromatography from ovomucin-removed egg white. J. Chromatogr. B Analyt. Technol.
586 Biomed. Life Sci. 878:1771–1776.

587 Omana DA, Liang Y, Kav NN, Wu J, (2011). Proteomic analysis of egg white proteins during
588 storage. Proteomics. 11:144– 153.

589 Pereira F, Carneiro J, Amorim A, (2008). Identification of Species with DNA-Based Technology:
590 Current Progress and Challenges. Recent Patents on DNA & Gene Sequences 2, 187-200.

591 Qiu N, Ma M, Zhao L, Liu W, Li Y, Yoshinori M, (2012). Comparative Proteomic Analysis of Egg
592 White Proteins under Various Storage Temperatures. J. Agric. Food Chem. 2012, 60,
593 7746−7753.

Raikos V, Hansen R, Campell L, Eusten SR, (2006). Separation and identification of hen egg protein isoforms using SDS–PAGE and 2D gel electrophoresis with MALDI-TOF mass spectrometry. Food Chemistry 99: 702–710.

Rath A, Glibowicka M, Nadeau VG, Chen G, Deber CM, (2009). Detergent binding explains anomalous SDS-PAGE migration of membrane proteins PNAS 106 (6): 1760 – 1765.

Stadelman WJ, Cotterill, OJ, (2001). Egg Science and Technology. 4th ed. Avi Publ. Co., Westport, CT.

Sunwoo HH, Gujral N, (2014). Chemical Composition of Egg and Egg Products. Handbook of Food Chemistry. DOI 10.1007/978-3-642-41609-5_28-1.

Wang J, Liang Y, Omana DA, Kav NN, Wu J. (2012). Proteomics Analysis of Egg White Proteins from Different Egg Varieties J. Agric. Food Chem. 60: 272−282.

Weiss W, Weiland F, Görg A, (2009). Protein detection and quantitation technologies for gel-based proteome analysis, in J. Reinders & A. Sickmann, eds, 'Proteomics', Vol. 564 of Methods in Molecular Biology, Humana Press.

Wellman-Labadie O, Picman J, Hincke MT, (2008). Comparative antibacterial activity of avian egg white protein extracts. Br Poult Sci. 49 (2):125-32.

Zheng, C, Ma G, Su Z, (2007). Native PAGE eliminates the problem of PEG–SDS interaction in SDS-PAGE and provides an alternative to HPLC in characterization of protein PEGylation. Electrophoresis 28: 2801–2807.